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(57) Abstract

A DNA construct comprising a DNA sequence homologous to some or all of a sequence encoding a xanthophyll biosynthetic enzyme or a xanthophyll degradative enzyme. Preferably, the DNA sequence encodes capsanthin-capsorubin synthase (CCS).

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DNA CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

The invention relates to novel DNA constructs, plant cells containing the constructs and plants derived therefrom.

In particular it relates to the modification of carotenoid metabolism in plants using DNA constructs.

The modification of plant gene expression has been achieved by several methods. The molecular biologist can choose from a range of known methods to decrease or increase gene expression or to alter the spatial or temporal expression of a particular gene. For example, the expression of either specific antisense RNA or partial (truncated) sense RNA has been utilized to reduce the expression of various target genes in plants (as reviewed by Bird and Ray, 1991, Biotechnology and Genetic Engineering, Reviews 9: 207-227). These techniques involve the incorporation into the genome of the plant of a synthetic gene designed to express either antisense or sense RNA. They have been successfully used to down-regulate the expression of a range of individual genes involved in the development and ripening of tomato fruit (Gray et al., 1992, Plant Molecular Biology, 19: 69-87).

Methods to increase the expression of a target gene have also been developed. For example, additional genes designed to express RNA containing the complete coding region of the target gene may be incorporated into the genome of the plant to "over-express" the gene product. Various other methods to modify gene expression are known; for example, the use of alternative regulatory sequences.

The carotenoid pathway in plants produces carotenes, lutein, xanthophylls, and pigments such as lycopene. An earlier patent application (published as EP-A-505405) describes a process to modify (inhibit or promote) the synthesis of such compounds in plants using DNA constructs comprising a DNA sequence preferably encoding a phytoene synthase enzyme (which particularly modifies colour of plant parts, especially fruit).

The late steps of carotenoid biosynthesis in plants involve the formation of xanthophylls. Little is known about the enzymology of these steps. No plant xanthophyll biosynthetic enzyme has previously been cloned. In work leading to the present invention we have purified to homogeneity a xanthophyll biosynthetic enzyme from *Capsicum annuum* (pepper) chromoplasts, which catalyzes the conversion of the ubiquitous 5,6-epoxycarotenoids, antheraxanthin and violaxanthin, into capsanthin and capsorubin, respectively. Due to its

bifunctionality, this new enzyme has been named capsanthin-capsorubin synthase (CCS).

Both capsanthin and capsorubin are red and give colour to plant tissue. Currently both of these xanthophylls (extracted from paprika) are used as food colourants. Capsanthin and capsorubin are unique to *Capsicum* fruits and CCS may only be naturally expressed in species of this genus (including peppers, chillies and paprika). However, the immediate precursors for capsanthin and capsorubin (violaxanthin and antheraxanthin) are present in all green tissues.

The present invention relates to a DNA sequence, containing:

- all or part of the nucleotide sequence represented on Figure 3, coding for a messenger RNA (mRNA), with said mRNA itself coding for a xanthophyll metabolic enzyme represented on Figure 3, designated by capsanthin-capsorubin synthase (CCS),

- all or part of any nucleotide sequence which is derived from the above-mentioned sequence represented on Figure 3, particularly by mutation and/or addition and/or substitution of one or several nucleotide(s), with this derived sequence coding for a mRNA itself coding for the enzyme represented on Figure 3, or for a protein derived from said enzyme and presenting an enzymatic activity which is equivalent to the activity of the xanthophyll metabolic enzyme of Figure 3 in plants.

The present invention also relates to a DNA sequence, containing:

- all or part of the complementary nucleotidic sequence of the one represented on Figure 3, and such as defined above, with this complementary sequence coding for an antisense mRNA capable of hybridizing with a mRNA as defined above, or

- all or part of any DNA sequence which is derived from the above-mentioned complementary sequence, particularly by mutation and/or addition and/or substitution of one or several nucleotide(s), with this derived sequence coding for an antisense mRNA capable of hybridizing with an mRNA as defined above.

The present invention also relates to a mRNA coded by a DNA sequence as defined above, and more particularly coded by the DNA sequence represented on Figure 3, with said mRNA being capable of coding itself all or part of the xanthophyll metabolic enzyme represented on Figure 3, or for all or part of a protein derived from this enzyme, and presenting an activity which is equivalent to said enzyme in plants.

The present invention also relates to an antisense mRNA comprising nucleotides which are complementary of all or part of the nucleotides

constituting a mRNA as defined above, and capable of hybridizing with said mRNA.

The present invention also relates to an antisense mRNA as defined above, characterized by the fact that it is coded by a DNA sequence as defined above, and by the fact that it is capable of hybridizing with the mRNA coded by the DNA sequence represented on Figure 3.

The present invention also relates to a CCS present in *Capsicum annuum* cells and such as represented on Figure 3, or any protein derived from said CCS, particularly by addition and/or suppression and/or substitution of one or several amino-acids, or any fragment from said CCS or derived sequence, with said fragments and derived sequences being capable of presenting an enzymatic activity equivalent to the one of CCS.

The present invention also relates to a nucleotidic sequence coding for the CCS represented on Figure 3, or any derived sequence or fragment from said CCS, as defined above, with said nucleotidic sequence being characterized by the fact that it corresponds to all or part of the sequence represented on Figure 3, or to any sequence which is derived from this latter by the degeneracy of the genetic code, and being capable of coding for the CCS, or a derived sequence, or a fragment from said CCS, such as defined above.

The present invention also relates to a complex formed between an antisense mRNA as defined above, and a mRNA as defined above, capable of coding for a CCS in plants.

The present invention also relates to a recombinant DNA characterized by the fact:

- that it comprises a DNA sequence as defined above, with said sequence according to claim 1 being inserted in a heterologous sequence capable of coding for mRNA itself capable of coding for CCS.

The present invention also relates to a recombinant DNA characterized by the fact:

- that it comprises a DNA sequence which is complementary of a DNA sequence as defined above, inserted in a heterologous sequence, with said complementary DNA sequence being able to code for an antisense mRNA capable of hybridizing with the mRNA coding for a CCS in plants.

The present invention also relates to a DNA recombinant as defined above, characterized by the fact that it comprises the elements necessary to control the expression of the nucleotidic sequence as defined above, or of its complementary sequence as defined above, particularly a promotor and a terminator of the transcription of said sequences.

The present invention also relates to a recombinant vector characterized by the fact that it comprises a recombinant DNA as defined above, integrated in one of its sites of its genome, which are non essential for its replication.

The present invention also relates to a process for modifying the production of carotenoid in plants, either by enhancing the production of carotenoid, or by lowering or inhibiting the production of the carotenoid by the plants, with respect to the normal contents of carotenoid produced by plants, said process comprising the transformation of cells of said plants, with a vector as defined above.

The present invention also relates to plants or fragments of plants, particularly fruits, seeds, leaves, petals or cells transformed by incorporation of at least one of the nucleotidic sequences as defined above, into their genome.

According to the present invention, there is provided a DNA construct comprising a DNA sequence homologous to some or all of a sequence encoding a xanthophyll metabolic enzyme. The DNA sequence may be derived from cDNA, from genomic DNA or may be synthesized *ab initio*. The metabolic enzyme may be a xanthophyll biosynthetic enzyme or a xanthophyll degradative enzyme. Preferably, the DNA sequence encodes capsanthin-capsorubin synthase (CCS).

The purified CCS enzyme is a monomer with a molecular mass of 50 kDa. Antibodies raised against this enzyme allowed the isolation of a full length cDNA clone encoding a capsanthin-capsorubin synthase high molecular weight precursor. The cDNA sequence is shown in Figure 3. The deduced primary structure reveals the presence of a consensus nucleotide binding site. The capsanthin-capsorubin synthase gene is specifically expressed during chromoplast development in fruits accumulating ketocarotenoids, but not in mutants impaired in this biosynthetic step.

cDNA clones encoding CCS or other xanthophyll metabolic enzyme may be obtained from cDNA libraries using standard methods. Sequences coding for the whole, or substantially the whole, of the mRNA produced by the corresponding gene may thus be obtained. The cDNA so obtained may be sequenced according to known methods.

An alternative source of the DNA sequence is a suitable gene encoding the appropriate xanthophyll metabolic enzyme. This gene may differ from the corresponding cDNA in that introns may be present. The introns are not transcribed into mRNA (or, if so transcribed, are subsequently cut out). Oligonucleotide probes or the cDNA clone may be used to isolate the actual xanthophyll metabolic enzyme gene(s) by screening genomic DNA libraries.

Such genomic clones may include control sequences operating in the plant genome. Thus it is also possible to isolate promoter sequences which may be used to drive expression of the enzymes or any other protein. These promoters may be particularly responsive to certain developmental events and environmental conditions. Xanthophyll metabolic enzyme gene promoters may be used to drive expression of any target gene.

A further way of obtaining a xanthophyll metabolic enzyme DNA sequence is to synthesize it *ab initio* from the appropriate bases, for example using the appropriate cDNA sequence as a guide (for example, Figure 3 for CCS).

It is clear that xanthophyll metabolic enzyme-encoding sequences may be isolated not only from *Capsicum* species but from any suitable plant species. Alternative sources of suitable genes include bacteria, yeast, lower and higher eukaryotes.

The xanthophyll metabolic enzyme-encoding sequences may be incorporated into DNA constructs suitable for plant transformation. These DNA constructs may then be used to modify gene expression in plants. "Antisense" or "partial sense" or other techniques may be used to reduce the expression of the xanthophyll metabolic enzyme(s) in plant tissue. The levels of the xanthophyll metabolic enzymes may also be increased; for example, by incorporation of additional enzyme genes. The additional genes may be designed to give either the same or different spatial and temporal patterns of expression in the plant.

The overall level of xanthophyll metabolic enzyme activity and the relative activities of the individual enzymes affect the development and final form of carotenoid content in the plant and thus determine certain characteristics of the plant parts. Modification of xanthophyll metabolic enzyme activity can therefore be used to modify various aspects of plant (including fruit) quality. The activity levels of the xanthophyll metabolic enzymes may be either reduced or increased during development depending on the characteristics desired for the modified plant. Enhancing expression of a biosynthetic enzyme will increase production of the particular xanthophyll product, and inhibiting expression will decrease production. Enhancing expression of a degradative enzyme will decrease levels of the xanthophyll being degraded, while inhibiting expression will increase levels of said xanthophyll.

For example, the down-regulation of CCS biosynthetic enzyme activity in peppers (eg using antisense or sense constructs) will inhibit capsorubin and/or capsanthin production to alter fruit colour. Such down-regulation may result in an accumulation of the immediate precursors of these red pigments,

antheraxanthin and violaxanthin, which are orange/yellow. As a further example, over-expression of CCS in *Capsicum* species may be used to enhance fruit colour.

CCS may also be expressed in cells, tissues and organisms that do not normally produce capsorubin or capsanthin. A DNA sense construct encoding and expressing the functional CCS enzyme may be transformed into any suitable eukaryotic or prokaryotic cell (plant, fungi, algae, bacteria, animal etc). As the immediate precursors for capsanthin and capsorubin are present in all green plant tissue, expression of the CCS enzyme in such tissue leads to capsanthin and capsorubin synthesis. In other cases, the introduction of additional carotenoid biosynthetic genes may be necessary to ensure a supply of the precursors.

CCS could be used to produce capsorubin and/or capsanthin in any higher plant (including *Capsicum* species, tomato, carrot, cabbage, etc) since the immediate precursors are ubiquitous. This may be useful to change or enhance the colour of the plant or organ depending on the promoter used to drive CCS. It is particularly useful for modifying fruit and vegetable colour but may equally be applied to leaves and other organs.

Capsorubin or capsanthin produced by a eukaryotic or prokaryotic organism expressing a CCS-encoding DNA construct may be extracted for use as a food colourant.

As a further aspect of the invention, a process for the production of capsorubin or capsanthin is provided which comprises transformation of a eukaryotic or prokaryotic cell with a DNA construct encoding and expressing a protein having CCS enzyme activity. It may be necessary to transform the cell with additional constructs expressing enzymes needed to produce the necessary precursors.

A process for the production of violaxanthin or antheraxanthin is further provided which comprises transformation of an eukaryotic or prokaryotic cell with a DNA construct encoding at least part of a protein having CCS enzyme activity so that production of capsorubin or capsanthin is inhibited.

The activity of the xanthophyll metabolic enzyme may be modified either individually or in combination with modification of the activity of another similar or unrelated enzyme. For example, the activity of the CCS enzyme may be modified in combination with modification of the activity of a cell wall enzyme involved in fruit ripening.

Use of the novel xanthophyll metabolic enzyme constructs provides a method for modification of plant characteristics comprising modification of the activity of xanthophyll metabolic enzymes.

According to the present invention there is further provided a DNA construct comprising a DNA sequence homologous to some or all of a sequence encoding a xanthophyll metabolic enzyme under the control of a transcriptional initiation region operative in plants, so that the construct can generate RNA in plant cells.

The characteristics of plant parts (particularly fruit) may be modified by transformation with a DNA construct according to the invention. The invention also provides plant cells containing such constructs; plants derived therefrom showing modified fruit characteristics; and seeds of such plants.

A DNA construct according to the invention may be an "antisense" construct generating "antisense" RNA or "sense" construct (encoding at least part of the functional enzyme) generating "sense" RNA. "Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). "Sense RNA" is an RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence. Such sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). Suitable sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO 91/08299) or to over-express the enzyme.

The constructs of the invention may be inserted into plants to regulate the production of xanthophyll metabolic enzymes. The constructs may be transformed into any dicotyledonous or monocotyledonous plant. Depending on the nature of the construct, the production of the enzyme may be increased or reduced, either throughout or at particular stages in the life of the plant. Generally, as would be expected, production of the enzyme is enhanced only by

constructs wich express RNA homologous to the substantially complete endogenous enzyme mRNAs. Full-length sense constructs may also inhibit enzyme expression. Constructs containing an incomplete DNA sequence shorter than that corresponding to the complete gene generally inhibit the expression of the gene and production of the enzymes, whether they are arranged to express sense or antisense RNA.

Full-length antisense constructs also inhibit gene expression.

In a DNA construct according to the invention, the transcriptional initiation region may be derived from any plant-operative promoter. The transcriptional initiation region may be positioned for transcription of a DNA sequence encoding RNA which is complementary to a substantial run of bases in a mRNA encoding the xanthophyll metabolic enzyme (making the DNA construct a full or partial antisense construct).

DNA constructs according to the invention may comprise a base sequence at least 10 bases (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA or synthetic polynucleotide may be used. The isolation of suitable xanthophyll metabolic biosynthetic enzyme-encoding sequences is described above. Sequences coding for the whole, or substantially the whole, of the appropriate enzyme may thus be obtained. Suitable lengths of these DNA sequences may be cut out for use by means of restriction enzymes. When using genomic DNA as the source of a partial base sequence for transcription it is possible to use either intron or exon regions or a combination of both.

To obtain constructs suitable for expression of the appropriate xanthophyll metabolic enzyme sequence in plant cells, the cDNA sequence as found in the enzyme cDNA or the gene sequence as found in the chromosome of the plant may be used. Recombinant DNA constructs may be made using standard techniques. For example, the DNA sequence for transcription may be obtained by treating a vector containing said sequence with restriction enzymes to cut out the appropriate segment. The DNA sequence for transcription may also be generated by annealing and ligating synthetic oligonucleotides or by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to give suitable restriction sites at each end. The DNA sequence is then cloned into a vector containing upstream promoter and downstream terminator sequences. If

antisense DNA is required, the cloning is carried out so that the cut DNA sequence is inverted with respect to its orientation in the strand from which it was cut.

In a construct expressing antisense RNA, the strand that was formerly the template strand becomes the coding strand, and vice versa. The construct will thus encode RNA in a base sequence which is complementary to part or all of the sequence of the enzyme mRNA. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5' to 3').

In a construct expressing sense RNA, the template and coding strands retain the assignments and orientations of the original plant gene. Constructs expressing sense RNA encode RNA with a base sequence which is homologous to part or all of the sequence of the mRNA. In constructs which express the functional enzyme, the whole of the coding region of the gene is linked to transcriptional control sequences capable of expression in plants.

For example, constructs according to the present invention may be made as follows. A suitable vector containing the desired base sequence for transcription (such as the CCS cDNA clone) is treated with restriction enzymes to cut the sequence out. The DNA strand so obtained is cloned (if desired, in reverse orientation) into a second vector containing the desired promoter sequence and the desired terminator sequence. Suitable promoters include the 35S cauliflower mosaic virus promoter and the tomato polygalacturonase gene promoter sequence (Bird et al., 1988, Plant Molecular Biology, **11**: 651-662) or other developmentally regulated fruit promoters. Suitable terminator sequences include that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end).

The transcriptional initiation region (or promoter) operative in plants may be a constitutive promoter (such as the 35S cauliflower mosaic virus promoter) or an inducible or developmentally regulated promoter (such as fruit-specific promoters), as circumstances require. For example, it may be desirable to modify enzyme activity only during fruit development and/or ripening. Use of a constitutive promoter will tend to affect enzyme levels and functions in all parts of the plant, while use of a tissue specific promoter allows more selective control of gene expression and affected functions (eg fruit colouration). Thus in applying the invention (for example, to peppers) it may be found convenient to use a promoter that will give expression during fruit development and/or ripening. Thus the antisense or sense RNA is only produced in the organ in which its action is required. Fruit development and/or ripening-specific

promoters that could be used include the ripening-enhanced polygalacturonase promoter (International Patent Publication Number WO 92/08798), the E8 promoter (Diekman & Fischer, 1988, EMBO, 7: 3315-3320) and the fruit specific 2A11 promoter (Pear et al., 1989, Plant Molecular Biology, 13: 639-651).

Carotenoid (particularly xanthophyll) content (and hence plant characteristics) may be modified to a greater or lesser extent by controlling the degree of the appropriate xanthophyll metabolic enzyme's sense or antisense mRNA production in the plant cells. This may be done by suitable choice of promoter sequences, or by selecting the number of copies or the site of integration of the DNA sequences that are introduced into the plant genome. For example, the DNA construct may include more than one DNA sequence encoding the xanthophyll metabolic enzyme or more than one recombinant construct may be transformed into each plant cell.

The activity of a first xanthophyll metabolic enzyme may be separately modified by transformation with a suitable DNA construct comprising a DNA sequence encoding the first enzyme. The activity of a second xanthophyll metabolic enzyme may be separately modified by transformation with a suitable DNA construct comprising a DNA sequence encoding the second enzyme. In addition, the activity of both the first and second enzymes may be simultaneaously modified by transforming a cell with two separate constructs: the first comprising a first enzyme-encoding sequence and the second comprising a second enzyme-encoding sequence. Alternatively, a plant cell may be transformed with a single DNA construct comprising both a first enzyme-encoding sequence and a second enzyme-encoding sequence.

It is also possible to modify the activity of the xanthophyll metabolic enzymes while also modifying the activity of one or more other enzymes. For example, the other enzymes may be involved in cell metabolism or in fruit development and ripening. Other cell wall metabolising enzymes that may be modified in combination with xanthophyll metabolic enzymes include but are not limited to: pectin esterase, polygalacturonase, β-galactanase, β-glucanase. Other enzymes involved in fruit development and ripening that may be modified in combination with xanthophyll metabolic enzymes include but are not limited to: ethylene biosynthetic enzymes, other carotenoid biosynthetic enzymes including phytoene synthase, carbohydrate metabolism enzymes including invertase.

Several methods are available for modification of the activity of the xanthophyll metabolic enzymes in combination with other enzymes. For example, a first plant may be individually transformed with a CCS construct and

then crossed with a second plant which has been individually transformed with a construct encoding another enzyme. As a further example, plants may be either consecutively or co-transformed with CCS constructs and with appropriate constructs for modification of the activity of the other enzyme(s). An alternative example is plant transformation with a CCS construct which itself contains an additional gene for modification of the activity of the other enzyme(s). The xanthophyll metabolic biosynthetic enzyme constructs may contain sequences of DNA for regulation of the expression of the other enzyme(s) located adjacent to the xanthophyll enzyme sequences. These additional sequences may be in either sense or antisense orientation as described in International Patent Application Publication number WO 93/23551 (single construct having distinct DNA regions homologous to different target genes). By using such methods, the benefits of modifying the activity of the xanthophyll metabolic enzymes may be combined with the benefits of modifying the activity of other enzymes.

A DNA construct of the invention is transformed into a target plant cell. The target plant cell may be part of a whole plant or may be an isolated cell or part of a tissue which may be regenerated into a whole plant. The target plant cell may be selected from any monocotyledonous or dicotyledonous plant species. Suitable plants include any fruit-bearing plant (such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, peppers, chillies, paprika). For any particular plant cell, the xanthophyll metabolic enzyme sequence used in the transformation construct may be derived from the same plant species, or may be derived from any other plant species (sufficient sequence similarity to allow modification of related enzyme gene expression).

Constructs according to the invention may be used to transform any plant using any suitable transformation technique to make plants according to the invention. Both monocotyledonous and dicotyledonous plant cells may be transformed in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Any suitable method of plant transformation may be used. For example, dicotyledonous plants such as tomato and melon may be transformed by *Agrobacterium* Ti plasmid technology, such as described by Bevan (1984, Nucleic Acid Research, 12: 8711-8721) or Fillatti et al. (Biotechnology, July 1987, 5: 726-730). Such transformed plants may be reproduced sexually, or by cell or tissue culture.

A process for modifying the production of carotenoids in plants is further provided by transforming such plants with DNA adapted to modify carotenoid

biosynthesis and growing such transformed plants or their descendants to produce plant parts (for example leaves, petals or fruit) of modified carotenoid content. Suitable DNA comprises, *inter alia*, constructs according to the present invention, but other similar constructs affecting other parts of the carotenoid pathway may also be used. Such constructs may be adapted to enhance the production of carotenoids (for example xanthophylls) or inhibit such production by the plant.

As well as colour production, other important functions may be modified by the process of the invention. Thus β-carotene (a precursor of Vitamin A) and other carotenoids are important to human health, and have been claimed to have a protective effect against certain diseases. Food plants may be modified by transformation with the constructs of the invention so that they have a higher content of such compounds: or other plants may be so modified, so that they can act as a source from which such compounds can be extracted. Carotenoids are also believed to have a role in protecting plants against high light intensity damage, so plants with a higher content of such compounds may be of value in combating the effects of any global climate change.

In this way, plants can be generated which have modified colour due to promotion or inhibition of the pathways of carotenoid biosynthesis. In particular, CCS constructs may be used to promote or inhibit the production of the red colour associated with capsorubin or capsanthin. For example, inhibition of this red colour in peppers (eg by transformation with antisense or sense constructs) may give fruit of an attractive shade of orange or yellow. Similar orange/yellow peppers are known, but the present invention provides means of transferring the trait into elite lines without a prolonged breeding programme which might alter other traits at the same time. Promotion of capsorubin or capsanthin production (eg by sense over-expression constructs) may produce peppers of a deeper red colour, which may appear more appetising to the consumer.

The invention may also be used to introduce a red colour into parts of plants other than the fruit. For example, promotion of capsorubin or capsanthin may be brought about by inserting one or more functional copies of the gene cDNA, or of the full-length gene, under control of a promoter functional in plants. If capsorubin or capsanthin are naturally expressed in the plant, the promoter may be selected to give a higher degree of expression than is given by the natural promoter.

Examples of genetically modified plants according to the present invention include fruit-bearing plants. The fruit of such plants may be made more

attractive (or at least interesting) by inducing or intensifying a red colour therein. Other plants that may be modified by the process of the invention include tubers such as radishes, turnips and potatoes, as well as cereals such as maize (corn), wheat, barley and rice. Flowers of modified colour, and ornamental grasses either red or reddish overall, or having red seedheads, may be produced.

As already discussed, plants produced by the process of the invention may also contain other recombinant constructs, for example constructs having other effects on fruit ripening. For example fruit of enhanced colour according to the invention may also contain constructs inhibiting the production of enzymes such as polygalacturonase and pectinesterase, or interfering with ethylene production. Fruit containing both types of recombinant construct may be made either by successive transformations, or by crossing two varieties that each contain one of the constructs, and selecting among the progeny for those that contain both.

The invention will now be described by way of example only, with reference to the drawings in which:

Figure 1 is a diagram of the proposed reaction and mechanism for the biosynthesis of capsanthin and capsorubin; [conversion of antheraxanthin and violaxanthin into capsanthin and capsorubin; the reaction occurs through a cationic attack and the resulting carbocation is stabilized by ejection of a proton];

Figure 2 is a graph of the HPLC analysis of the conversion of antheraxanthin into capsanthin by chromoplast achlorophyllous membranes; [The HPLC profile of the achlorophyllous membrane pigments obtained after incubation and addition of unlabelled carrier antheraxanthin and capsanthin, followed by the distribution of incorporated radioactivity using (b) boiled purified membranes (25 μg) and (c) unboiled isolated membranes (25 μg), separately incubated with (^{14}C) antheraxanthin as indicated in Example 1. After 1h at 28°C, the lipid extract was subjected to HPLC analysis. The position of the different xanthophylls is indicated: (1) neoxanthin; (2) capsorubin; (3) violaxanthin; (4) capsanthin; (5) antheraxanthin].

Figure 3 shows the nucleotide and deduced amino acid sequence of the *C. annuum* capsanthin-capsorubin cDNA; [A potential dinucleotide binding site (G*G**G***A******G) is underlined. Numberings are given in the left margin for amino acids and above the sequence for nucleotides].

Background to the invention

To explore the molecular characteristics of the individual plant carotenogenic enzymes, we have used *Capsicum annuum* chromoplasts as a biological model (Carmara and Monéger, 1982; Camara et al., 1989). This system has allowed the characterization and cloning of geranylgeranyl pyrophosphate synthase (GGPPS) (Dogbo and Camara, 1987; Kuntz et al., 1992), phytoene synthase (Dogbo et al., 1988; Römer et al., 1993) and phytoene-phytofluene desaturase (Hugueney et al., 1992). Concerning the latter steps of the plant carotenogenic pathway which involves the synthesis of xanthophylls, much less is known. Several lines of evidence (Cholnoky et al., 1955; Cholnoky et al. 1955; Davies et al., 1970; Neamtu and Bodea, 1969; Neamtu et al., 1969; Valadon and Mummery, 1977) suggest that epoxyxanthophylls are reactive intermediates involved in the enzymatic interconversion of carotenoids (Costes et al., 1979; Camara, 1980a,b; Camara and Monéger, 1980; Camara and Monéger, 1981) as part of the xanthophyll cycle (Yamamoto, 1979; Yamamoto and Higashi, 1978) and even in the formation of abscisic acid and related derivatives (Rock and Zeevart, 1991; Li and Walton, 1990). However, practically nothing is known about the enzymology of xanthophyll biogenesis.

In ealier studies (Camara, 1980a,b; Camara and Monéger, 1980; Camara and Monéger, 1981), we demonstrated that the ubiquitous 5,6-epoxyxanthophylls, antheraxanthin and violaxanthin, are the direct precursors of capsanthin and capsorubin, respectively, according to the scheme depicted in Figure 1. The characteristics of these ketoxanthophyll synthases are largely unknown. Further development of a refined *in vitro* system that is mediating this activity would be a critical step toward defining the molecular characteristics of these xanthophyll biosynthetic enzymes and offer the potential of modifying the later steps of plant carotenogenesis. In Example 1, we describe the properties of such a system, which has allowed the purification of the monomeric and multifunctional xanthophyll biosynthetic enzyme capsanthin-capsorubin synthase (CCS) and the cloning of its cDNA using antibodies directed against this enzyme. We show that the CCS gene is specifically regulated during chloroplast to chromoplast differentiation.

EXAMPLE 1

Subplastidial compartmentation of capsanthin-capsorubin synthase

At the final stage of differentiation, *C. annuum* chromoplasts are composed of two permanent entities, the soluble fraction (stroma) and the membrane fraction. The latter comprises the plastid envelope as well as a set of new membranes, including achlorophyllous inner membranes and fibrils, both of which are not present in chloroplasts (Spurr and Harris, 1968; Camara and Brangeon, 1981). We have demonstrated previously that isopentenyl pyrophosphate is converted into phytoene by stromal enzymes while the latter steps which involve phytoene desaturation, cyclization and oxidation, are catalysed by membrane-bound enzymes (Camara et al., 1982; Camara et al., 1985). Since the plastid envelope membrane is present in all type of plastids, while the inner achlorophyllous membranes and the fibrils are specifically and strongly triggered during chloroplast to chromoplast differentiation, we hypothesized that the ketoxanthophyll synthase(s) involved in the active biogenesis of capsanthin and capsorubin was most likely associated with the inner achlorophyllous membranes and/or the fibrils which have been shown to be the main site of carotenoid accumulation in *C. annuum* chromoplasts (Deruère et al., 1994). Therefore, we decided to develop a procedure for the purification of these two chromoplast sub-fractions as described under "Experimental procedures". Electron microscope analysis clearly indicated that these purified fibrils and membrane fractions showed negligible cross-contamination. Previously, we showed that 95% of the chromoplast carotenoids are compartmentalized in the fibrils (Deruère et al., 1994). HPLC analysis of the purified achlorophyllous membranes revealed that qualitatively their major carotenoids are capsanthin and capsorubin while epoxyxanthophylls are minor components.

To test their potential ketoxanthophyll synthase activity, both fractions were incubated with labelled antheraxanthin. The data displayed in Figure 2 indicate that this enzymic activity is associated with the purified membrane fraction. Only a negligible activity (less than 2%) was present in the fibrils. We were unable to separate these intraplastidial membranes from the plastid envelope membranes and assess their potential activity. However, plastid envelope membranes or thylakoid membranes prepared from fruit chloroplasts according to Douce and Joyard (1979), were unable to carry out this enzymic conversion (results not shown).

Purification and characteristics of capsanthin-capsorubin synthase

Compared with whole plastid membranes, the purified membrane fraction had a less complex protein pattern. Based on these data, we selected the chromoplast membrane fraction as the starting material for the purification of the ketoxanthophyll synthase(s). Preliminary experiments were carried out to solubilize the ketoxanthophyll synthase(s) from *C. annuum* membranes. Treatment of the membranes with 1% Triton X-100 or 1% octylglucoside readily solubilized 90% of the ketoxanthophyll synthase initially present in the membrane fraction. Owing to its higher critical micelle concentration, we selected octylglucoside for the subsequent purification steps.

Further analysis of the purified fraction using SDS-PAGE showed one band with an approximate molecular mass of 50 kDa. Also after an additional chromatography on a calibrated column of Sephacryl S-200, a native molecular mass of 60 kDa was determined. Therefore, we conclude that the active enzyme is most probably a monomer. The different purification steps are summarized in Table 1.

The purified enzyme shows a pale yellow colour and gives an intense fluorescence, suggesting that it could be a flavoprotein. However, further spectral analysis and attempts to remove the prosthetic group by acidic pH were inconclusive. Further analysis are required to determine the nature of the prosthetic group.

It has been shown previously that the two 5,6-epoxyxanthophylls antheraxanthin and violaxanthin are the precursors of capsanthin and capsorubin, respectively (Camara, 1980a,b; Camara and Monéger, 1980; Camara and Monéger, 1981). Therefore, an important question remains: does the same enzyme catalyze the formation of both ketoxanthophylls? To address this question, the purified enzyme was incubated with (^{14}C) violaxanthin under different experimental conditions as indicated in Table 2. The data obtained indicate that the purified ketoxanthophyll synthase does indeed catalyze the conversion of violaxanthin into capsorubin. Parallel studies aimed at identifying an additional enzyme, immunologically distinct from this ketoxanthophyll synthase, which catalyzes only the conversion of violaxanthin into capsorubin failed. On the basis of this finding, we propose the name capsanthin-capsorubin synthase (CCS) for this ketoxanthophyll synthase which, like other carotenogenic enzymes already characterized from *C. annuum*, i.e. GGPPS (Dogbo and Camara, 1987), phytoene synthase (Dogbo et al., 1988) and phytoene desaturase (Hugueney et al., 1992), is a multifunctional protein. One can suggest that this type of molecular organization probably results in a better

channeling of the substrates which, starting from the prenyl pyrophosphates, are supposed to move from one block of modifying enzymes to another until the final step of the pathway.

Immunological characteristics of capsanthin-capsorubin synthase

Antibodies directed against CCS revealed a single polypeptide of 50 kD specifically localized in the chromoplast membrane fraction. No signal was obtained for chloroplast thylakoid membranes or chloroplast envelope membranes. Only a negligible signal was observed in fibrils.

A solubilized plastid extract was treated with preimmune or anti-CCS serum and the immune complexes were precipitated with protein-A Sepharose according to a previously described procedure (Dogbo et al., 1987). The enzymatic activity of the supernatant fluid was determined. Only anti-CCS antibodies gave a concentration-dependent inhibition (results not shown), thus giving an additional criterium of specificity. Finally, immunocytochemical analysis revealed that CCS is strictly compartmentalized in the plastid. From these data, the serum was considered to be sufficiently specific for developmental studies and for screening a pepper fruit cDNA library.

Isolation of a capsanthin-capsorubin synthase cDNA

Immunoscreening of a *C. annuum* cDNA library allowed isolation of 2 positive clones of about approximately 300 and 1700 bp, respectively. Subcloning and sequencing of these cDNAs revealed that the 300 bp cDNA is a fragment of the 1700 bp cDNA and that common sequences are completely identical. The larger cDNA possesses an open-reading frame of 498 codons (Figure 3). The fact that this open-reading frame is preceded by a stop codon in the same frame suggests that this cDNA contains the whole coding sequence.

The deduced amino acid sequence corresponds to a protein of 57 kDa. As expected for a plastid-targeted protein, the NH_2-terminus of this protein resembles a typical transit peptide which probably ends before the acidic amino acid-rich region which starts at position 56. Cleavage of the transit peptide a few amino acids before this position would leave a mature protein of approximately 50 kDa, in good agreement with the size of the purified CCS estimated by SDS-PAGE.

A search through the sequence databanks revealed no significant sequence similarity between the deduced CCS sequence and other known sequences, except for a 17 amino acid motif close to the NH_2-terminus of the mature protein which resembles a typical dinucleotide binding site (Carothers et al.,

1989). Such a motif has also been observed by several authors in the NH_2-terminal portion of plant and bacterial phytoene desaturases (Bartley et al., 1991; Hugueney et al., 1992; Misawa et al., 1990; Pecker et al., 1992). Further examination of the CCS sequence revealed several short motifs which are also present in the bacterial lycopene cyclases (not shown), in addition to the motif FLYAXXPXXXXXXXLXE which is present in bacterial zeaxanthin synthase (Misawa et al., 1990). The presence of the latter motif could be due to the fact that zeaxanthin synthase like capsanthin-capsorubin synthase acts on β-cyclohexenyl ring (Camara, 1980a).

Capsanthin-capsorubin synthase is specifically synthesized during chromoplast differentiation

Western blot analysis during *C. annuum* development indicated that the synthesis of CCS synthase is specifically and strongly induced during chromoplast differentiation. In addition, RNA gel blot analysis using the CCS cDNA as a probe revealed that the corresponding gene is not expressed in leaves and green fruits from *C. annuum* but only in ripening fruits. During ripening, the expression pattern of the CCS gene appears to be similar to that of the GGPPS gene, namely a strong induction at the early ripening stage which is maintained up to the ripe stage. To confirm this observation, other RNA samples (Kuntz et al., 1992), harvested independently from fruits from a different plant (at a mature green stage, an intermediate ripening stage and a fully ripe stage), were used. Here again, the same pattern of expression was found (data not shown) for both the CCS and the GGPPS gene, namely a strong induction maintained throughout ripening and a decline in the RNA steady-state level later in the fully ripe fruit. These observations suggest that these genes are coexpressed during fruit ripening in *C. annuum*, although the GGPPS gene, unlike the CCS gene, is expressed at low level in all carotenogenic tissues (Kuntz et al., 1992).

Occurrence of capsanthin-capsorubin synthase in fruit colour mutants

To extend the above-mentioned data, we made use of different fruit colour mutants of *C. annuum*. In particular, it was of interest to see whether CCS is absent in the yellow cultivars Jaune de Pignerolle or Golden Summer in which the pericarp turns from green to yellow at the final ripening stage, in the cultivar Sweet Chocolate in which the pericarp turns brown during the final stage of ripening, in the cultivar Alma in which the red colour involves the differentiation of a proplastid into a chromoplast and in the cultivar Permagreen

which remains green at the final stage of ripening. Toward this goal, we first analyzed their xanthophyll content by HPLC. The chromatograms obtained indicate the absence of capsanthin and capsorubin in the green and yellow fruit mutants. Similar results were obtained for the cultivar Permagreen at the full ripening stage. In contrast, these ketoxanthophylls are present at the final steps of ripening in the Sweet Chocolate mutant and in the red fruits.

Additional immunological analysis of the green fruits and of yellow mutants revealed that CCS is completely absent. In contrast, CCS is actively synthesized in the Sweet Chocolate mutant and in all red ripe fruits. From these data, we conclude that the yellow fruit mutants and the cultivar Permagreen are specifically impaired in CCS.

RNA samples from ripening fruits of different *C. annuum* varieties were also probed for the presence of the CCS transcript. As shown in Figure 10b and d, CCS gene expression could not be detected in the yellow fruit cultivars Jaune de Pignerolle and Golden Summer (even after over-exposure of the autoradiographs). In contrast, the GGPPS gene was found to be expressed in both cultivars. These data are in agreement with the absence of the CCS protein in these cultivars.

The data presented indicate that CCS is a chromoplast-specific protein. We have previously characterized another chromoplast-specific protein that we termed fibrillin and which is the major protein associated with chromoplast fibrils (Deruère et al., 1994). In the fibrillar type of chromoplasts (e.g. in *C. annuum* fruits or *Palisota barteri* fruits, Knoth et al., 1986), fibrils are lipoprotein structures where most of the carotenoids are stored. In contrast, CCS is found in a different chromoplast sub-compartment, namely in membrane structures and most likely in the inner achlorophyllous membranes. These observations suggest that carotenoids once synthesized are discharged into their site of storage (fibrils). Whether this phenomenon involves additional proteins is debatable. Further analysis of the deduced peptide sequence of CCS reveals that the sequence EEKCVIT is similar to the neurofilament consensus sequence EEKVVVTK which is probably involved in several cellular interactions (Hisanaga and Hirokawa, 1988; Shaw, 1992). Therefore, one could suggest that this domain helps in connecting the achlorophyllous membranes network displayed by silver proteinate post-staining and/or mediates a direct interaction between CCS and fibrillin. This data is reminiscent to previous results on cholinesterase, indicating that an enzyme could have a structural role in addition to its catalytic activity (Krejci et al. 1991).

Chromoplast-specific proteins have also been observed by other authors (Winkenbach et al., 1976; Hadjeb et al., 1988; Smirra et al., 1993). One of them, termed ChrA, has a molecular mass of 58 kD and is a carotenoid binding protein (Cervantes-Cervantes et al., 1990). However, when the complete CCS amino acid sequence was compared with the partial amino acid sequence deduced from the published sequence of the ChrA gene (Oren-Shamir et al., 1993), the best homology obtained was only continuous stretch of 10 amino acids. Furthermore, no significant sequence similarity was found between the CCS cDNA and the ChrA gene, which makes it unlikely that CCS is encoded by the published ChrA gene. It should also be mentioned that no transcript corresponding to ChrA could be detected in red *C. annuum* fruits (Oren-Shamir et al., 1993).

Conclusion

A plant xanthophyll biosynthetic enzyme has been isolated which catalyzes two late steps of the carotenogenic pathway, namely the synthesis of capsanthin and capsorubin, and cloned its cDNA. To our knowledge, a previous xanthophyll biosynthetic enzyme has not been described in details, nor its cDNA cloned. The data reported here affords a tool for the molecular analysis of the role of 5,6-epoxyxanthophylls.

Genetics studies (Atkins and Sherrard, 1915; Hurtado-Hernandez and Smith, 1985) have previously assigned the red colour of *C. annuum* fruits to the *y* locus. Genetic characterization of this locus is currently in progress in order to determine whether *y* encode(s) CCS or is pleiotropic on the CCS gene.

Experimental procedures

Substrate preparation

(^{14}C) antheraxanthin and (^{14}C) violaxanthin were prepared using *Capsicum annuum* pericarp disk of the yellow cultivar Jaune de Pignerolle incubated overnight with (2-^{14}C) potassium mevalonate (60 mCi/mmol, Amersham, France) according to a previously described procedure (Camara, 1980a).

Plant material

Bell pepper plants (*Capsicum annuum* cv. Yolo Wonder) were grown under controlled greenhouse conditions until the fruit ripened, as indicated by the red colour. Several other fruit colour mutants were also grown under the

same conditions. These included cultivar Sweet Chocolate which turns into a chocolate colour due to the presence of a chlorophyll retainer gene, cultivar Alma in which the red colour involves proplastid to chromoplast differentiation, yellow cultivars in which the green fruit turns into a deep yellow (cv. Jaune de Pignerolle) or light yellow (cv. Golden Summer) colour, and cultivar Permagreen which remains green at the final stage of ripening.

Plastid subfractionation and enzyme preparations

annuum chromoplasts, prepared from 1 kg of red fruit according to a previously described procedure (Camara, 1993), were osmotically lysed by direct resuspension into 100 ml of 50 mM Tris-HCl buffer pH 7.6 containing 1 mM DTT, followed by homogenization using a Potter homogenizer. Twenty ml of the resulting homogenate were layered onto a discontinuous sucrose gradient (identical volumes of 0.5 and 0.9 M sucrose) in the same buffer supplemented with 1 mM EDTA. After 1h centrifugation at 70 000 g at 4°C, using a SW27 rotor (Beckman), the whole fraction above the 0.9 M layer was recovered and loaded onto the top of a 0 to 1 M linear sucrose gradient in the same buffer, and was centrifuged in a SW27 rotor. After centrifugation at 70 000 g for 12h at 4°C, the chromoplast fibrils banded at a density of 1.07 g/ml while the purified membrane fraction was recovered at a density corresponding to 1.10 g/ml. The typical yield of purified membranes varied from 400 to 500 μg per kg of fruit pericarp. To 2.8 mg of the purified chromoplast membranes, prepared as described above and resuspended in 5 ml 50 mM Tris-HCl pH 8 (buffer A), were added an equal volume of 2% Triton X-100 or 2% octylglucoside dissolved in the same buffer, in order to solubilize the membrane-bound enzyme. The mixture was stirred at 5°C for 30 min before centrifugation at 100 000 g for 1h. The supernatant containing the solubilized enzyme was recovered for further use.

Enzymic assay

The assay mixture in a total volume of 0.25 ml contained: a definite amount of enzyme extract (^{14}C) antheraxanthin or (^{14}C) violaxanthin (100 000 cpm per μmole) dissolved in 10 μl of ethanol, 250 μg of stromal extract (Hugueney et al., 1992), 0.2 mM $NADP^+$, 0.2 mM NADPH, 1 mM ATP, buffered with 50 mM Tris HCl pH 7.6. After incubation at 28°C for 30 min, unlabelled xanthophyll standards were added before extraction and analysis of the reaction products by HPLC (Camara, 1985), and determination of the incorporated radioactivity by liquid scintillation.

Enzyme purification

Q-Sepharose chromatography: the solubilisate obtained as described above was batch-adsorbed onto Q-Sepharose (Pharmacia) equilibrated with buffer A containing 0.5% octylglucoside (buffer B). After washing with buffer B containing 25 mM NaCl, the active fraction was eluted in bulk with buffer B containing 0.3 M NaCl. After ten-fold dilution, the active fraction was then adsorbed onto a Q-Sepharose column (2 x 20 cm) equilibrated with buffer B containing 50 mM NaCl. The active fractions were then eluted with a linear salt gradient (0 to 0.3 M NaCl) in buffer B. Five ml fractions were collected for the determination of enzyme activity and protein content using the dye binding method (Bio-Rad protein assay kit).

Affigel 501 chromatography: pooled active fractions from the Q-Sepharose column were loaded onto a Affigel 501 column (1 x 10 cm, Bio-Rad) previously equilibrated with buffer B. The flow through fraction was discarded and after washing the column with ten volumes of buffer B, the active fraction was eluted in bulk by adding 10 mM DTT in buffer B.

Mono P chromatography: the active fraction from the Affigel 501 column was applied onto a Mono P column (HR 5/20, Pharmacia) equilibrated with buffer A. The active fractions were eluted with 12.5% polybuffer 96 (pH 5). Fractions of 1 ml were collected for enzymic assay and protein determination.

Gel filtration: active fractions after the chromatofocusing steps were concentrated by ultrafiltration and applied to a Sephacryl S-200 column (1.5 x 90 cm, Pharmacia) previously equilibrated with buffer B containing 0.1 M NaCl. Fractions of 2.5 ml were collected for enzymic assay and protein determination. The same column was used for the molecular mass determination, using several calibrated molecular mass standards.

Immunoscreening, RNA and DNA techniques

Construction of a λgt11 cDNA library with RNA isolated from a *C. annuum* fruit at an early ripening stage was described previously (Kuntz et al., 1992). Previously described procedures were used for screening, RNA and DNA work (see Kuntz et al., 1992). Hybridizations were performed using standard procedures at 65°C in 2 x SSC. Membranes were then washed at 65°C in 0.5 x SSC.

Microscopy and immunohistochemistry

Pericarp tissue or plastid subfractions were treated as described previously (Camara, 1993; Deruère et al., 1994).

Analytical techniques

The lipid extract was saponified and the xanthophyll fraction obtained after filtration through a silica cartridge was used for HPLC. The pigments were eluted isocratically using methanol/acetone/water (90: 17: 3), as decribed previously (Camara, 1985). Radioactivity was determined by liquid scintillation counting.

SDS-PAGE and immunoblotting were carried out as described previously (Kuntz et al., 1992) using antibodies directed against the purified enzyme, according to standard techniques. Protein determinations were carried out using a Bio-Rad protein assay kit.

Table 1. Purification of ketoxanthophyll synthase from *C. annuum* chromoplast membranes

Steps	Total protein	Total activity	Specific activity (nmole/h/mg protein)	Recovery (%)
Membranes	2.8	294	105	100
Solubilized membranes	2	252	126	86
Q-Sepharose	0.8	163	204	56
Affigel 501	0.4	148	370	50
Mono-P	0.2	85	425	29
Sephacryl	0.18	79	439	27

Table 2. Enzymic conversion of violaxanthin into capsorubin

Substrate	Radioactivity incorporated into capsorubin by boiled membranes	Radioactivity incorporated into capsorubin by native membranes
Violaxanthin (15 000 cpm)	25	3200
Violaxanthin (25 000 cpm)	36	6750

Purified chromoplast membranes (25 μg) were incubated with the indicated amount of (^{14}C) violaxanthin as indicated under Experimental Procedure, before analysis of the radioactivity incorporated in capsorubin. A boiled membrane preparation was used as a control.

The CCS cDNA, or a related DNA fragment as defined in the present invention, can be used as a molecular marker to facilitate plant breeding programs. For instance, isolation of genomic DNA from seedlings produced during plant breeding programs (after sexual or somatic crosses between the same species or different species) and molecular hybridization of the CCS cDNA (or a related DNA fragment) to this genomic DNA can identify those seedlings which possess to CCS gene. This will allow to predict the phenotype of these plants (regarding the fruit colour) without having to wait for fruit formation and ripening.

EXAMPLE 2

Construction of antisense RNA vectors with the CaMV 35S promoter.

A vector is constructed using the sequences corresponding to a fragment of the insert of a CCS cDNA (isolated as shown in example 1). This fragment is synthesized by polymerase chain reaction using synthetic primers. The ends of the fragment are made flush with T4 polymerase and it is cloned into the vector pJR1 which has previously been cut with SmaI. pJR1 (Smith et al., 1988, Nature, 334: 724-726) is a Bin19 (Bevan, 1984, Nucleic Acids Research, 12: 8711-8721) based vector, which permits the expression of the antisense RNA under the control of the CaMV 35S promoter. This vector includes a nopaline synthase (nos) 3' end termination sequence.

Alternatively a vector is constructed using a restriction fragment obtained from a CCS cDNA and cloned into the vectors GA643 (An et al., 1988, Plant Molecular Biology Manual A3: 1-19) or pDH51 (Pietrzak et al., 1986, Nucleic Acids Research, **14**: 5875-5869) which has previously been cut with a compatible restriction enzyme(s). A restriction fragment from the CCS/pDH51 clone containing the promoter, the CCS fragment and other pDH51 sequence is cloned into SLJ44026B or SLJ44024B (Jones et al., 1990, Transgenic Research, 1) or Bin19 (Bevan, 1984, Nucleic Acids Research, **12**: 8711-8721) which permits the expression of the antisense RNA under control of the CaMV 35S promoter.

After synthesis of the vector, the structure and orientation of the sequences are confirmed by DNA sequence analysis.

EXAMPLE 3

Construction of antisense RNA vectors with a fruit-enhanced promoter.

The fragment of the CCS cDNA that was described in Example 2 is also cloned into the vector pJR3. pJR3 is a Bin19 based vector, which permits the expression of the antisense RNA under the control of the tomato polygalacturonase (PG) promoter. This vector includes approximately 5 kb of promoter sequence and 1.8 kb of 3' sequence from the PG promoter separated by a multiple cloning site.

After synthesis, vectors with the correct orientation of the CCS sequences are identified by DNA sequence analysis.

Alternative fruit-enhanced promoters (such as E8 or 2A11) are substituted for the polygalacturonase promoter in pJR3 to give alternative patterns of expression.

EXAMPLE 4

Construction of truncated sense RNA vectors with the CaMV 35S promoter.

The fragment of the CCS cDNA that was described in Example 2 is also cloned into the vectors described in Example 2 in the sense orientation.

After synthesis, the vectors with the sense orientation of the CCS sequence are identified by DNA sequence analysis.

EXAMPLE 5

Construction of truncated sense RNA vectors with fruit-enhanced promoter.

The fragment of the CCS cDNA that was described in Example 2 is also cloned into the vector pJR3 in the sense orientation.

After synthesis, the vectors with the sense orientation of the CCS sequence are identified by DNA sequence analysis.

Alternative fruit-enhanced promoters (eg E8 or 2A11) are substituted for the polygalacturonase promoter in pJR3 to give alternative patterns of expression.

EXAMPLE 6

Construction of a CCS over-expression vector using the CaMV 35S promoter.

The complete sequence of a CCS cDNA containing a full open-reading frame is inserted into the vectors described in Example 2.

EXAMPLE 7

Construction of a CCS over-expression vector using a fruit-enhanced promoter.

The complete sequence of a CCS cDNA containing a full open-reading frame is inserted into pJR3 or alternatives with different promoters.

EXAMPLE 8

Generation of transformed plants.

Vectors are transferred to *Agrobacterium tumefaciens* LBA4404 (a micro-organism widely available to plant biotechnologists) and are used to transform tomato plants. Transformation of tomato stem segments follow standard protocols (e.g. Bird et al., 1988, Plant Molecular Biology, **11**: 651-662). Transformed plants are identified by their ability to grow on media containing the antibiotic kanamycin. Plants are regenerated and grown to maturity. Ripening fruits are analysed for modifications to their ripening characteristics.

EXAMPLE 9

It is to be noted that the CCS cDNA, or restriction fragments of the CCS cDNA, or DNA fragments derived from the CCS cDNA, or oligonucleotides designed from the CCS nucleotide sequence, or oligonucleotides designed from the CCS amino acid sequence can be used to isolate other cDNAs or genomic DNA sequences. These cDNAs or genomic DNAs can be isolated directly from cDNA or genomic libraries using the above-mentioned DNA molecules as hybridization probes. Alternatively, a combination of the above-mentioned oligonucleotides can be used to generate DNA fragments by polymerase chain reactions (PCR).

The isolated DNAs code for enzymes or portion of enzymes which catalyze chemical reactions similar to the one shown in Figure 1. Examples of such enzymes are cyclases (such as those catalyzing the synthesis of α-carotene or β-carotene), oxidases (introduction of hydroxyl groups, keto groups, aldehyde groups, epoxide groups) and de-epoxidases.

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EMBL Data Library Accession number X76165 (capsanthin-capsorubin synthase, *Capsicum annuum*).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE
(B) STREET: 3, rue Michel-Ange
(C) CITY: PARIS
(E) COUNTRY: FRANCE
(F) POSTAL CODE (ZIP): F-75016

(ii) TITLE OF INVENTION: DNA CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 94400626.1

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1756 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 67..1560

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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       Met Glu Thr Leu Leu Lys Pro Phe Pro Ser Pro Leu Leu Ser
         1               5                  10

ATT CCT ACT CCT AAC ATG TAT AGT TTC AAA CAC AAC TCC ACT TTT CCA       156
Ile Pro Thr Pro Asn Met Tyr Ser Phe Lys His Asn Ser Thr Phe Pro
 15                  20                  25                  30

AAT CCA ACC AAA CAA AAA GAT TCA AGA AAG TTC CAT TAT AGA AAC AAA       204
Asn Pro Thr Lys Gln Lys Asp Ser Arg Lys Phe His Tyr Arg Asn Lys
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CCA GAG TCT TTA GAT GTT AAC ATC TCA TGG GTT GAT ACT GAT CTG GAC      300
Pro Glu Ser Leu Asp Val Asn Ile Ser Trp Val Asp Thr Asp Leu Asp
        65                  70                  75

GGG GCT GAA TTC GAC GTG ATC ATC ATT GGA ACT GGC CCT GCC GGG CTT      348
Gly Ala Glu Phe Asp Val Ile Ile Ile Gly Thr Gly Pro Ala Gly Leu
    80                  85                  90

CGG CTA GCT GAA CAA GTT TCT AAA TAT GGT ATT AAG GTA TGT TGC GTT      396
Arg Leu Ala Glu Gln Val Ser Lys Tyr Gly Ile Lys Val Cys Cys Val
95                  100                 105                 110

GAC CCT TCA CCA CTT TCC ATG TGG CCA AAT AAT TAT GGT GTT TGG GTT      444
Asp Pro Ser Pro Leu Ser Met Trp Pro Asn Asn Tyr Gly Val Trp Val
                115                 120                 125

GAT GAG TTT GAA AAG TTG GGA TTA GAA GAT TGT CTA GAT CAT AAG TGG      492
Asp Glu Phe Glu Lys Leu Gly Leu Glu Asp Cys Leu Asp His Lys Trp
            130                 135                 140

CCT GTG AGT TGT GTT CAT ATA AGT GAT CAC AAG ACT AAG TAT TTG GAC      540
Pro Val Ser Cys Val His Ile Ser Asp His Lys Thr Lys Tyr Leu Asp
        145                 150                 155

AGA CCA TAT GGT AGA GTA AGT AGA AAG AAG TTG AAG TTG AAA TTG TTG      588
Arg Pro Tyr Gly Arg Val Ser Arg Lys Lys Leu Lys Leu Lys Leu Leu
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Asn Ser Cys Val Glu Asn Arg Val Lys Phe Tyr Lys Ala Lys Val Leu
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AAA GTG AAG CAT GAA GAA TTT GAG TCT TCG ATT GTT TGT GAT GAT GGT      684
Lys Val Lys His Glu Glu Phe Glu Ser Ser Ile Val Cys Asp Asp Gly
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AGG AAG ATA AGC GGT AGC TTG ATT GTT GAT GCA AGT GGC TAT GCT AGT      732
Arg Lys Ile Ser Gly Ser Leu Ile Val Asp Ala Ser Gly Tyr Ala Ser
            210                 215                 220

GAT TTT ATA GAG TAT GAC AAG CCA AGA AAC CAT GGT TAT CAA GTT GCT      780
Asp Phe Ile Glu Tyr Asp Lys Pro Arg Asn His Gly Tyr Gln Val Ala
        225                 230                 235

CAT GGG ATT TTA GCA GAA GTT GAT AAT CAT CCA TTT GAT TTG GAT AAA      828
His Gly Ile Leu Ala Glu Val Asp Asn His Pro Phe Asp Leu Asp Lys
    240                 245                 250

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            290                 295                 300

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Pro Met Leu Ser Tyr Met Glu Val Lys Arg Arg Met Val Ala Arg Leu
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Arg His Leu Gly Ile Lys Val Arg Ser Val Leu Glu Glu Glu Lys Cys
    320                 325                 330

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Val Ile Thr Met Gly Gly Pro Leu Pro Arg Ile Pro Gln Asn Val Met
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GCT ATT GGT GGG ACT TCA GGG ATA GTT CAT CCA TCG TCT GGG TAC ATG     1164
Ala Ile Gly Gly Thr Ser Gly Ile Val His Pro Ser Ser Gly Tyr Met
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Val Ala Arg Ser Met Ala Leu Ala Pro Val Leu Ala Glu Ala Ile Val
            370                 375                 380

GAA AGC CTT GGC TCA ACA AGA ATG ATA AGA GGG TCT CAA CTT TAC CAT     1260
Glu Ser Leu Gly Ser Thr Arg Met Ile Arg Gly Ser Gln Leu Tyr His
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Arg Val Trp Asn Gly Leu Trp Pro Ser Asp Arg Arg Arg Val Arg Glu
    400                 405                 410

TGT TAT TGT TTC GGA ATG GAG ACT TTG TTG AAG CTT GAT TTG GAA GGT     1356
Cys Tyr Cys Phe Gly Met Glu Thr Leu Leu Lys Leu Asp Leu Glu Gly
415                 420                 425                 430

ACT AGG AGA TTG TTT GAT GCT TTC TTT GAT GTT GAT CCC AAG TAC TGG     1404
Thr Arg Arg Leu Phe Asp Ala Phe Phe Asp Val Asp Pro Lys Tyr Trp
                435                 440                 445

CAC GGG TTC CTT TCT TCA AGA TTG TCT GTC AAA GAA CTT GCT GTA CTC     1452
His Gly Phe Leu Ser Ser Arg Leu Ser Val Lys Glu Leu Ala Val Leu
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 498 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Thr Lys Gln Lys Asp Ser Arg Lys Phe His Tyr Arg Asn Lys Ser Ser
35 40 45

Thr His Phe Cys Ser Phe Leu Asp Leu Ala Pro Thr Ser Lys Pro Glu
50 55 60

Ser Leu Asp Val Asn Ile Ser Trp Val Asp Thr Asp Leu Asp Gly Ala
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Glu Phe Asp Val Ile Ile Ile Gly Thr Gly Pro Ala Gly Leu Arg Leu
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Ala Glu Gln Val Ser Lys Tyr Gly Ile Lys Val Cys Cys Val Asp Pro
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Ser Pro Leu Ser Met Trp Pro Asn Asn Tyr Gly Val Trp Val Asp Glu
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Lys Cys Thr Val Pro Leu Val Lys Leu Leu Gly Asn Leu Ala Ile Glu
485 490 495

Ser Leu

CLAIMS

1. DNA sequence, containing:

- all or part of the nucleotide sequence represented on Figure 3, coding for a messenger RNA (mRNA), said mRNA itself coding for a xanthophyll metabolic enzyme represented on Figure 3, designated by capsanthin-capsorubin synthase (CCS).

- all or part of any nucleotide sequence which is derived from the above-mentioned sequence represented on Figure 3, particularly by mutation and/or addition and/or substitution of one or several nucleotide(s), with this derived sequence coding for a mRNA itself coding for the enzyme represented on Figure 3, or for a protein derived from said enzyme and presenting an enzymatic activity which is equivalent to the activity of the xanthophyll metabolic enzyme of Figure 3 in plants.

2. DNA sequence, containing:

- all or part of the complementary nucleotidic sequence of the one represented on Figure 3, and such as defined in claim 1, with this complementary sequence coding for an antisense mRNA capable of hybridizing with a mRNA according to claim 1, or

- all or part of any DNA sequence which is derived from the above-mentioned complementary sequence, particularly by mutation and/or addition and/or substitution of one or several nucleotide(s), with this derived sequence coding for an antisense mRNA capable of hybridizing with an mRNA according to claim 1.

3. mRNA coded by a DNA sequence according to claim 1, and more particularly coded by the DNA sequence represented on Figure 3, with said mRNA being capable of coding itself all or part of the xanthophyll metabolic enzyme represented on Figure 3, or for all or part of a protein derived from this enzyme, and presenting an activity which is equivalent to said enzyme in plants.

4. Antisense mRNA comprising nucleotides which are complementary of all or part of the nucleotides constituting a mRNA according to claim 3, and capable of hybridizing with said mRNA.

5. Antisense mRNA according to claim 4, characterized by the fact that it is coded by a DNA sequence according to claim 2, and by the fact that it is

capable of hybridizing with the mRNA coded by the DNA sequence represented on Figure 3.

6. CCS present in *Capsicum annuum* cells and such as represented on Figure 3, or any protein derived from said CCS, particularly by addition and/or suppression and/or substitution of one or several amino-acids, or any fragment from said CCS or derived sequence, with said fragments and derived sequences being capable of presenting an enzymatic activity equivalent to the one of CCS.

7. Nucleotidic sequence coding for the CCS represented on Figure 3, or any derived sequence or fragment from said CCS, according to claim 6, with said nucleotidic sequence being characterized by the fact that it corresponds to all or part of the sequence represented on Figure 3, or to any sequence which is derived from this latter by the degeneracy of the genetic code, and being capable of coding for the CCS, or a derived sequence, or a fragment from said CCS, such as defined in claim 6.

8. Complex formed between an antisense mRNA according to claim 4 or 5, and a mRNA according to claim 1, capable of coding for a CCS in plants.

9. Recombinant DNA characterized by the fact

- that it comprises a DNA sequence according to claim 1, with said sequence according to claim 1 being inserted in a heterologous sequence capable of coding for mRNA itself capable of coding for CCS, or
- by the fact that it comprises a DNA sequence which is complementary of a DNA sequence according to claim 1, inserted in a heterologous sequence, with said complementary DNA sequence being able to code for an antisense mRNA capable of hybridizing with the mRNA coding for a CCS in plants.

10. DNA recombinant according to claim 9, characterized by the fact that it comprises the elements necessary to control the expression of the nucleotidic sequence according to claim 1, or of its complementary sequence according to claim 2, particularly a promotor and a terminator of the transcription of said sequences.

11. Recombinant vector characterized by the fact that it comprises a recombinant DNA according to claims 9 or 10, integrated in one of its sites of its genome, which are non essential for its replication.

12. Process for modifying the production of carotenoid in plants, either by enhancing the production of carotenoid, or by lowering or inhibiting the production of the carotenoid by the plants, with respect to the normal contents of carotenoid produced by plants, said process comprising the transformation of cells of said plants, with a vector according to claim 11.

13. Plants or fragments of plants, particularly fruits, seeds, leaves, petals or cells transformed by incorporation of at least one of the nucleotidic sequences according to claim 1 or 2, into their genome.

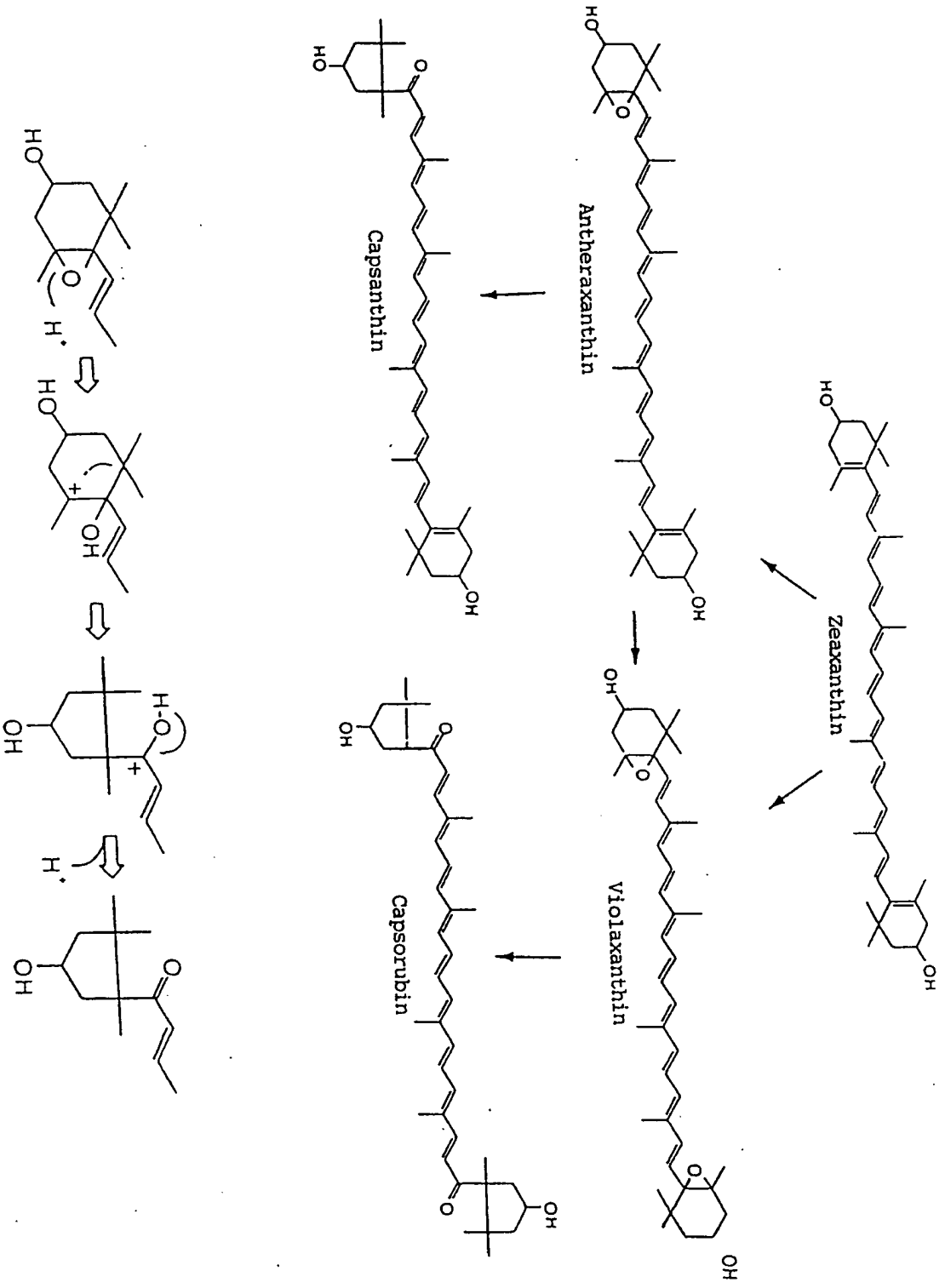

Capsanthin
Antheraxanthin
Zeaxanthin
Capsorubin
Violaxanthin

FIGURE 1

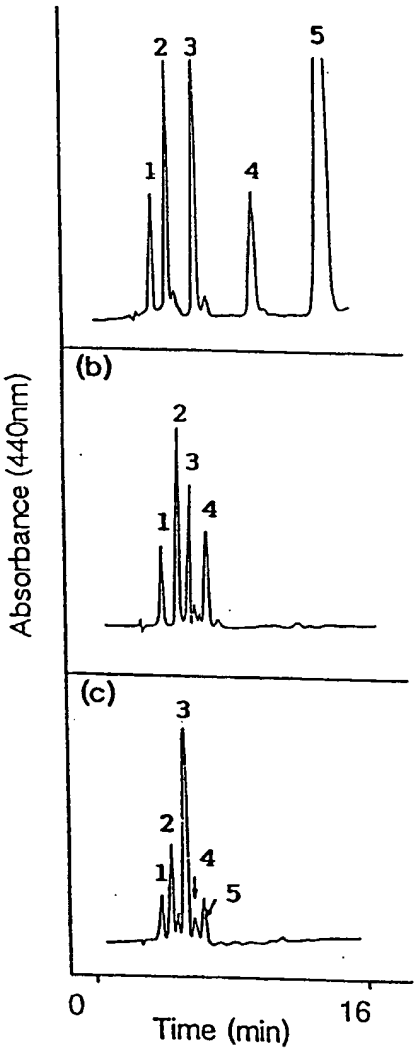
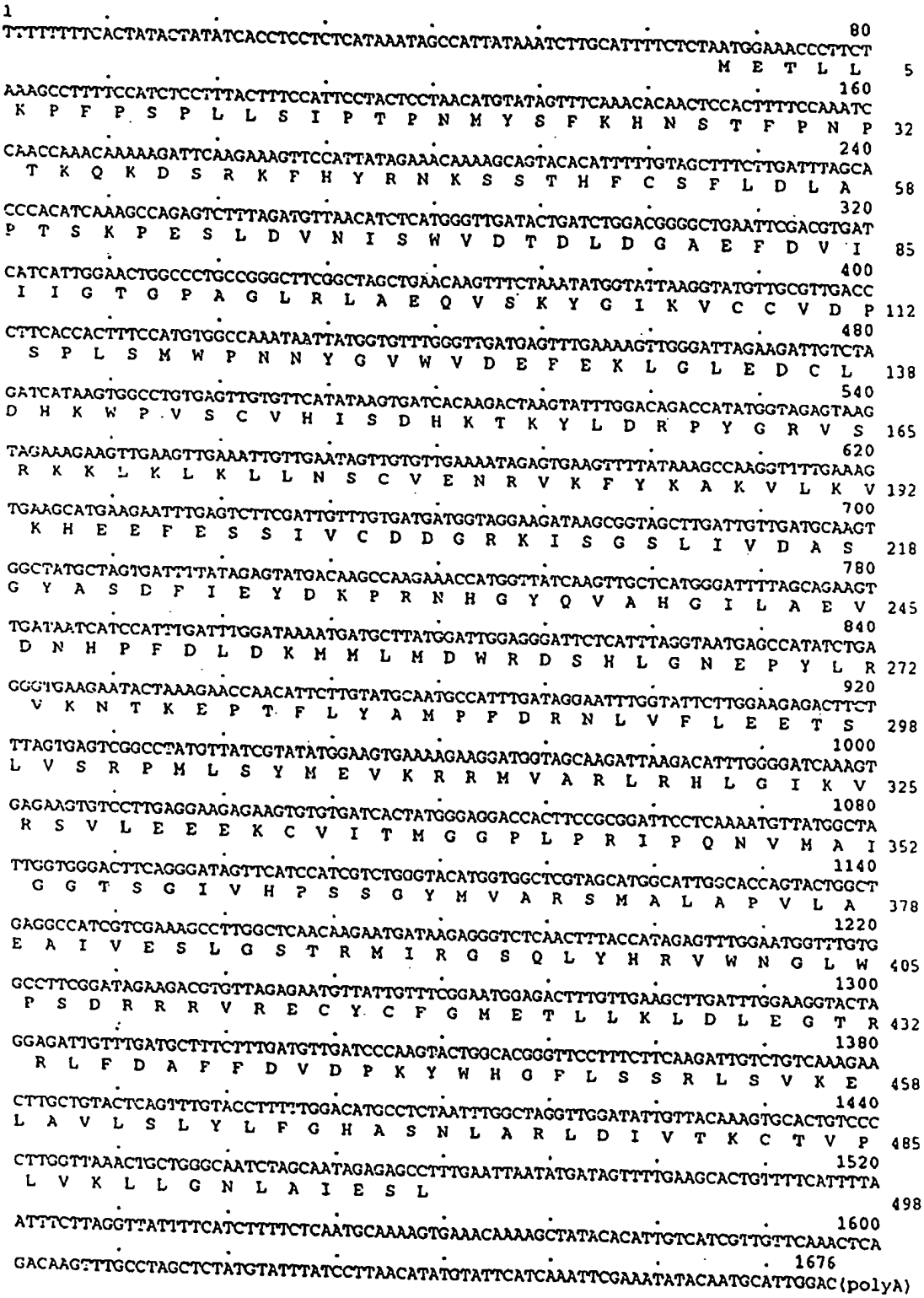


FIGURE 2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GGAGATTGTTTGATGCTTTCTTTGATGTTGATCCCAAGTACTGGCACGGGTTCCTTTCTTCAAGATTGTCTGTCAAAGAA
1380
R L F D A F F D V D P K Y W H G F L S S R L S V K E 458
CTTGCTGTACTCAGTTTGTACCTTTTTGGACATGCCTCTAATTTGGCTAGGTTGGATATTGTTACAAAGTGCACTGTCCC
1440
L A V L S L Y L F G H A S N L A R L D I V T K C T V P 485
CTTGGTTAAACTGCTGGGCAATCTAGCAATAGAGAGCCTTTGAATTAATATGATAGTTTTGAAGCACTGTTTTCATTTTA
1520
L V K L L G N L A I E S L
498
ATTTCTTAGGTTATTTTCATCTTTTCTCAATGCAAAAGTGAAACAAAAGCTATACACATTGTCATCGTTGTTCAAACTCA
1600
GACAAGTTTGCCTAGCTCTATGTATTTATCCTTAACATATGTATTCATCAAATTCGAAATATACAATGCATTGGAC(polyA)
1676

Figure 3

INTERNATIONAL SEARCH REPORT

Inte. ional Application No
PCT/EP 95/00584

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/82 C12N9/00 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 103, no. 7, 1985 Columbus, Ohio, US; abstract no. 50158, CAMARA, B., ET AL. 'Carotenogenic enzymes from Capsicum chromoplasts' see abstract & PURE APPL. CHEM., vol. 57, no. 5, 1985 pages 675-677, --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
8 June 1995	20.06.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

Inte. ional Application No
PCT/EP 95/00584

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	EMBL SEQUENCE DATABASE RELEASE 38 ACC. NO. Z29211. 2-1-94. DESPREZ,T., ET AL A.THALIANA TRANSCRIBED SEQUENCE. CLONE VBVYH06 see sequence ---	1,3,6,7
A	CHEMICAL ABSTRACTS, vol. 92, no. 19, 1980 Columbus, Ohio, US; abstract no. 160634, CAMARA, B. 'Carotenoid biosynthesis in vitro conversion of violaxanthin to capsorubin by a chromoplast enriched fraction of capsicum fruits' see abstract & BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 93, no. 1, 1980 DULUTH, MINNESOTA US, pages 113-117, ---	1-13
A	CHEMICAL ABSTRACTS, vol. 94, no. 23, 1981 Columbus, Ohio, US; abstract no. 188728, CAMARA, B. 'Carotenoid biosynthesis: in vitro conversion of antheraxanthin to capsanthin by a chromoplast-enriched fraction of Capsicum fruits' see abstract & BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 99, no. 4, 1981 DULUTH, MINNESOTA US, pages 1117-1122, --- -/--	1-13

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Inte. ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOLOGICAL ABSTRACTS, vol. 95, 1993 Philadelphia, PA, US; abstract no. 9314, HUGUENEY, P., ET AL. 'Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and zeta carotene in Capsicum chromoplasts' see abstract & EUR. J. BIOCHEM., vol. 209, no. 1, 1992 pages 399-407, ---	1-13
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A	WO-A-91 13078 (AMOCO) 5 September 1991 see the whole document ---	1-13
A	PLANT MOLECULAR BIOLOGY, vol. 22, 1993 pages 589-602, FRAY, R., ET AL. 'Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression' see the whole document ---	1-13
A	BIOTECHNOLOGY, vol. 9, no. 7, July 1991 NEW YORK US, pages 635-639, BIRD, C.R., ET AL. 'Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes' see the whole document ---	1-13
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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 95/00584

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 90, no. 1, 1979 Columbus, Ohio, US; abstract no. 2253, YAMAMOTO, H.Y., ET AL. 'Violaxanthin de-epoxidase. Lipid composition and substrate specificity' see abstract & ARCH. BIOCHEM. BIOPHYS., vol. 190, no. 2, 1978 pages 514-522, -----	1-13

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ..tional Application No
PCT/EP 95/00584

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		AU-A-	6893891	18-07-91
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		JP-T-	5502160	22-04-93
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		JP-T-	5504686	22-07-93